High inhibition of Paenibacillus larvae and Listeria monocytogenes by Enterococcus isolated from different sources in Tunisia and identification of their bacteriocin genes

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High inhibition of *Paenibacillus larvae* and *Listeria monocytogenes* by *Enterococcus* isolated from different sources in Tunisia and identification of their bacteriocin genes

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Running head: Inhibition of *P. larvae* and *L.monocytogenes* by *Enterococcus*

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Significance and Impact of Study: Enterococci possess interesting properties not only for the food industry, but also for animal and human health. The antimicrobial potential of these bacteria includes principally bacteriocin-like molecules. With the aim of identifying bacteriocinogenic strains, a collection of 300 enterococci isolated from different origins were screened and their spectrum of action, as well as the gene encoding the bacteriocin, were determined. Fifty nine bacteriocin-producing *Enterococcus* showed high activity against *Listeria monocytogenes* and *Paenibacillus larvae*, the causative agent of American Foulbrood. Enterocin A, P and L50A/B were found in various combinations. The most important finding of this study is the growth inhibition of *P. larvae* due to bacteriocin-producing *Enterococcus*, which opens up the possibility to use these strains to control the disease in honeybees.

Abstract

A total of 300 isolates of *Enterococcus*, from different sources including feces of poultry, cow and sheep, raw milk, ricotta cheese, and water, in Tunisia, were screened for their antibacterial activity. Amongst them, 59 bacteriocin-producing strains were detected and identified by molecular methods. Genes encoding for *ent*A, *ent*P, *ent*B, *ent*L50A/B, AS-48, *bac*31 bacteriocins were targeted by PCR. The bacteriocin-producing strains were assigned to the species *Enterococcus faecium*, *Ent. faecalis*, *Ent. hirae*, *Ent. mundtii* and *Ent. durans*, respectively 34, 19, 3, 2 and 1 isolates. Antimicrobial activity was specifically observed against different spoilage and pathogenic microorganisms, such as *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *Escherichia coli*, *Ent. faecalis*, *Staphylococcus aureus*, *Salmonella enterica serovar* Enteritidis and *Paenibacillus larvae*. The inhibitory activity was totally lost after proteinase K treatment, thereby revealing the proteinaceous nature of the antimicrobial compound. Only three bacteriocin genes, namely *entP*, *entA*, and *ent*L50A/B were detected in the isolates included in this study. Enterocin A and P were the most frequent genes and they were found in 55 (93.2%) and 39 isolates (66.1%), respectively, followed by enterocin

L50A/B present in 27 isolates (45.7%). These newly identified bacteriocin-producing enterococci have the potential to be used in bio-preservation of food as well as biological control of foulbrood disease.

Keywords: *Enterococcus*; bacteriocin; *Paenibacillus larvae*; *Listeria monocytogenes*; Tunisia

Introduction

Enterococcus are Gram-positive, facultative anaerobic bacteria, belonging to the Lactic Acid Bacteria (LAB) group and they can be isolated from different ecosystems such as humans, animals, water, soil, plants, food, waste and poultry (Byappanahalli *et al*. 2012). The bacteria of the genus *Enterococcus* have the ability to produce several antimicrobial substances, including bacteriocins, specifically enterocins, which have the potential to be used in bio-preservation (Khan *et al*. 2010). According to Franz *et al*. (2007), enterocins can be divided into four classes: class I of lantibiotic enterocins, class II of small and non-lantibiotic peptides, class III of cyclic enterocins and class IV of large proteins. Bacteriocin-producing enterococci are widespread in nature. They have been isolated from numerous sources, such as dairy products (Foulquié *et al*. 2006), fermented sausages (Cocolin *et al*. 2007), fish (Bourouni *et al*. 2012), vegetables (Bennik *et al*. 1998; Zendo *et al*. 2005) and mammalian gastrointestinal tract (Carina Audisio *et al*. 2000; Brandão *et al.* 2010).

To date, the following enterocins from genus *Enterococcus* have been characterized: enterocin A (Aymerich *et al.* 1996), enterocin B (Casaus *et al*. 1997), enterocin P (Cintas *et al*. 1997), enterocins L50A and L50B (Cintas *et al.* 1998), enterocin I (identical to enterocin L50A, Floriano *et al.* 1998), enterocin Q (Cintas *et al*. 2000), enterocin M (a new variant of enterocin P, Mareková and Lauková 2002), enterocin CRL35 (Farías *et al*. 1996), bacteriocin 31 (Tomita *et al*. 1996), and mundticin (Bennik *et al*. 1998). In general, most enterocins belong to the group of class IIa bacteriocins, which are thermostable and pediocin-like bacteriocins. These are of considerable interest as biopreservatives, because of their high antilisterial activity (Galvez *et al*. 2007).

American Foulbrood (AFB) is a severe disease, affecting larvae of the honeybee *Apis mellifera*, caused by the spore-forming bacteria *P. larvae*. AFB is worldwide distributed and

significantly affects honeybee populations and production (Genersch 2010). Besides having a strong impact on beekeeping industry, pollination, productivity of agricultural systems and conservation of natural ecosystems may be also affected by AFB. Antibiotic treatments appear as an alternative to the burning of diseased bee colonies. Currently, the only antibiotic approved for prevention and control of AFB is oxytetracycline; however, there is evidence of oxytetracycline-resistant isolates of *P. larvae* in certain endemic areas. Moreover, several alternative treatments being used for control of this disease are plants extracts (Boligon *et al*. 2013), essential oils of various plants (Flesar *et al*. 2010; González *et al*. 2010) or propolis (Antunez *et al*. 2008; Bastos *et al*. 2008) and antagonistic bacteria such as *Bacillus thuringiensis* (Cherif *et al.* 2008), *Bacillus subtilis* (Sabaté *et al*. 2009), lactic acid bacteria isolated from fermented materials (Yoshiyama *et al*. 2013), and *Lactobacillus johnsonii* strains isolated from the gut of worker *Apis mellifera* L. bees (Carina Audisso *et al*. 2011).

The aim of this study was to screen for the production of antimicrobial compounds, as well as the identification of bacteriocin genes, a large collection of enterococcus isolates obtained from different sources, such as feces (from poultry, cow and sheep), water, ricotta cheese, and raw milk in Tunisia.

Results and Discussion

The interest on enterococci was raised in the last decades and most of the *in vitro* studies dealt with the ability of enterococci to produce bacteriocins, determining the spectrum of inhibitory activity, their physicochemical properties, and their application in food products.

In this study we investigated a collection of 300 enterococci isolated from different sources for their ability to produce bacteriocins. By using the agar well diffusion assay (AWDA), 59 (19.6%) enterococci isolates showed antibacterial activities (Table 1) against a large number of indicator microorganisms investigated. This result is similar to other reports studying bacteriocinogenic *Enterococcus* from animals, humans, and vegetables sources (Ozdemir *et*

al. 2011; Klibi *et al*. 2012; Birri *et al*. 2013), underlining the possibility that this trait is involved in the increase of the competitiveness of some *Enterococcus* isolates in specific ecological niches. After identification of the bacteriocinogenic isolates it was determined that 34 belonged to *Ent. faecium*, 19 to *Ent. faecalis*, 3 to *Ent. hirae*, 2 to *Ent. mundtii* and 1 to *Ent. durans*. The literature is rich of studies reporting antibacterial activities by these species as well as others (i.e. *Ent. columbae* and *Ent. casseliflavus*), not identified in our study (Butaye *et al*. 2002; Sabia *et al*. 2004; Poeta *et al*. 2007). Among the 59 strains, 21 *Ent. faecium*, 14 *Ent. faecalis*, 2 *Ent. hirae*, 1 *Ent. mundtii*, and 1 *Ent. durans* showed anti-*L. monocytogenes* activity. It is worth underlining that 13 isolates showed a large inhibition zone (>15mm). *L. ivanovii* and *L. innocua* were also inhibited by the majority of these isolates. The ability to inhibit the growth of *Listeria spp*. is common to most *Enterococcus* bacteriocins (Cocolin *et al*. 2007; Pinto *et al*. 2009; Rivas *et al*. 2012), this ability being related to the close phylogenetic relationship of enterococci and listeriae. *Enterococcus* strains which show antilisterial activity, due to bacteriocin production, are of great importance in food and dairy industries (Rodríguez *et al*. 2000; De Vuyst *et al*. 2003). Twenty-nine isolates showed an activity against *E. faecalis*, with a large inhibition zone exhibited by 18 enterococci isolates. Concerning *S. aureus*, 10 isolates exhibited an inhibition effect. These two species have been previously reported to be susceptible to enterococcal bacteriocins (Rivas *et al*. 2012). It is generally recognized that the inhibitory activity of enterococcal bacteriocin encompasses Gram-positive bacteria, including *L. monocytogenes*, but shows limited or no activity against Gram-negatives (Banwo *et al*. 2012), due to their outer membrane. However, we found that *S. enteritidis* and *E. coli* were inhibited by 7 and 16 *Enterococcus* isolates, respectively. Moreover, three *Enterococcus* isolates were able to inhibit simultaneously the both species, although the *in vitro* inhibition was not as remarkable as for other Gram-positive indicators. This finding has been rarely reported in bacteriocinogenic *Enterococcus* isolates (Todorov *et al*. 2010; Theppangna *et al*. 2007) and would have important perspectives in *Salmonella* control in animal production (Szabó *et al*. 2009) as well as food protection against pathogenic pathovars of *E. coli* (Sparo *et al*. 2013).

In our study, 36 isolates were able to inhibit *P. larvae*, however only for 25 of them it was possible to link this activity to the production of a bacteriocin by the AWDA. To the best of our knowledge, this is the second report on *P. larvae* growth inhibition due to bacteriocin-

producing *Enterococcus* strains (Yoshiyama *et al*. 2013), but the first report of *P. larvae* inhibition by *Enterococcus* strains isolated from non fermented feeds and foods, and other than *Ent. thailandicus*. It is of great interest to focus on the possible biotechnological application of this finding, especially when considering that *Enterococcus* genus is part of the microbiota of honeybee digestive tract (Carina Audisso *et al*. 2011). While it seems that through *in vitro* experiments the bacteriocin acts directly on *P. larvae* inducing the killing, during *in vivo* experiment attempting to use a bacteriocinogenic strains as probiotics for honeybees (Yoshiyama *et al*. 2013) another biological trait has been observed. Indeed, those authors reported that after oral administration of bacteriocin-producing isolates, including one *Ent. thailandicus*, to larvae and adults, the transcription levels of antimicrobial peptide genes, such as abaecin, defensin and hymenoptaecin, were found to increase significantly. It was suggested that bacteriocin-producing isolates stimulate the innate immune response in honeybees, which may be useful for preventing bacterial diseases in honeybees. The effects of bacteriocin-producing *Enterococcus* isolates on AFB infection *in vivo* should be further investigated to understand its full applicability to protect honeybees from this disease.

In our study, the antimicrobial activity exhibited by 59 *Enterococcus* isolates, was lost after proteinase K treatment, thereby revealing the proteinaceous nature of the substance responsible for the inhibition (Dal Bello *et al*. 2010). Some bacteriocins are produced on solid growth media, but not in liquid cultures (Qi *et al*. 2001). Indeed, the difference of inhibition activity detection in solid and liquid medium is explained by several reasons, firstly, adsorption of bacteriocin to the surface of bacteriocin producing strains. Secondly, necessity of physical contact between the producing strain and indicator strain in order to induce the production of bacteriocin. This control system allows energy savings in the bacterial cell (Riley 1998).

Three bacteriocin genes *entP*, *entA*, *ent*L50A/B were detected in this study. The structural genes of Enterocin A and P were shown to be the most frequent, being found in 55 (93.2%) and 39 isolates (66.1%), respectively. Lastly, enterocin L50A/B was found in 27 isolates (45.7%). Enterocin AS-48, *ent*B and *bac*31 genes were not found in any of the examined enterococci. For one isolate no amplification product was obtained by using the set of primers targeting the bactericion genes described in this study. Different combinations of bacteriocin-encoding genes were observed in 41 isolates: *ent*A+*ent*P+*ent*L50A/B (22 isolates), *ent*A+*ent*P (15 isolates), *ent*A+*ent*L50A/B (4 isolates). Enterocins A and P are grouped in the class II.1 bacteriocins of the pediocin family, which are apparently very effective in preventing the growth of *Listeria* (Eijsink *et al*. 1998, Banwo *et al*. 2012). The high prevalence of enterocins A and P have been reported previously, as well as enterocin B (Simonova *et al*. 2007; Rivas *et al*. 2012). In contrast to our findings, *ent*A and *ent*B were mainly reported to specifically occur concomitantly in *Ent. faecium* isolates (De Vuyst *et al*. 2003; Cocolin *et al*. 2007; Ozdemir *et al*. 2011). Combination of two, or more bacteriocin-encoding genes, have been reported previously (Sanchez *et al*. 2007; Strompfova *et al*. 2008) and this was also the case of our collection in which 41 strains contained 2 or 3 gene combinations (Table 1). The presence of three enterocin genes in 22 strains in our study indicates the potential of some strains to produce various bacteriocins. However, the occurrence of several enterocin structural genes in *Enterococcus* isolates does not always correlate with a higher bacteriocin activity in their supernatants, and not all enterocin genes are expressed at the same time (Casaus *et al*. 1997). It is worthy to note that no relationship could be established between the presence of enterocin structural genes and the activity spectra of these bacteriocinogenic isolates. Regarding the isolate for which no amplification was obtained using the bacteriocin primers selected in this study, it should be underlined that this indeed is not an efficient

producer of bacteriocins as it was only able to inhibit *L. innocua* and *P. larvae* by agar spot test and lost its activity after neutralization of the supernatant, supporting the fact that most probably the inhibition was caused by the production of acid compounds.

In conclusion, the most important finding of this study is the ability of bacteriocin-producing *Enterococcus* isolates to inhibit the growth of *P. larvae.* This outcome is very promising and might open a large horizon for the scientific community working on the control of AFB in honeybees. Certainly, *in vivo* experiments are needed to better understand how to exploit this finding.

Materials and Methods

Bacterial Strains

A collection of 300 *Enterococcus* isolates were obtained from different sources including feces of poultry, cow and sheep, water, ricotta cheese, and raw bovine milk. The enterococci were isolated on Slanetz Bartley agar (BioKar diagnostics, Allonne, France) and incubated at 37°C for 24-48h. Colonies with typical aspect of *Enterococcus* were isolated and incubated overnight at 37°C. Gram staining, catalase and oxidase tests were carried out to confirm the appurtenance of isolates to *Enterococcus* genera. The following bacteria were used as indicator strains: *Listeria* (*L*) *monocytogenes* ATCC 43256, *L. innocua* (our collection), *L. ivanovii* (our collection), *Escherichia* (*E*) *coli* ATCC 25922, *Enterococcus* (*Ent*) *faecalis* ATCC 29212, *Staphylococcus* (*S*) *aureus* ATCC 6538, *Salmonella enterica serovar* Enteritidis (*S. enteritidis*) (our collection), and *P. larvae* (kindly provided by Pr. Cherif Ameur).

Bacteriocin production assay

Bacteriocin activity was tested by the agar spot test (De Vuyst *et al.* 1996). Briefly, 50 µl of an overnight culture of the indicator strain was added to 5 ml Brain Heart Infusion (BHI)

broth (Oxoid, Italy) supplemented with 0.7% agar, mixed, and poured onto a BHI agar plate. A single colony of the isolate to be tested for antimicrobial activity was transferred with a sterile toothpick to the agar plate seeded with the indicator bacteria. Plates were incubated for 24 h at 37°C in aerobic conditions. The antimicrobial activity was visually detected by clear inhibition zones around the tested strain. A test of the agar well diffusion assay (AWDA) was also realized as following: 150 µl of the indicator culture was inoculated into 15 ml of BHI agar and poured into plates. Wells were punched in the agar and filled with cell-free supernatant extracts of tested *Enterococcus* isolates (100 µl), sterilized by filtration using 0.22 µm filters. The presence or absence of inhibition zones around the wells was determined after incubation for 24 h at 37 °C.

In order to confirm the proteinaceous nature of the antimicrobial compounds, cultures of the positive isolates were centrifuged at 8000 x g for 10 min at 4°C and supernatants were collected and adjusted to pH 7 with 1 mol l^{-1} NaOH to eliminate the effect due to organic acids and 100 µl were treated with 4 µl of proteinase K (25 mg ml^{-1}, Sigma). Both the neutralized and proteinase K treated supernatants (100 µl) were placed into a well in BHI agar plate containing 1% inoculum of an overnight culture of each of the indicator strains considered. The plates were then incubated at optimum growth temperature of the indicator strains and examined after 24 h for inhibition zone. The presence of inhibition zones around the well containing the neutralized culture broth and the absence of the halo around the well containing the proteinase K, confirmed the production of bacteriocins or bacteriocin-like compounds by the tested strains (Dal Bello *et al*. 2010).

Molecular identification of the bacteriocin-producing isolates

Strains showing antimicrobial activity were identified at the species level as described by Cocolin *et al*. (2004). First, the strains were subjected to a PCR reaction by using the primers

P1V1GC (5'-CGC CCG CCG CGC CCC GCG CCCGTC CCG CCG CCC CCG CCC GGC GGCGTG CCTAATACA TGC-3') and P2V1 (5'-TTC CCC ACG CGT TAC TCA CC-3'), and subsequently grouped by Denaturing Gradient Gel Electrophoresis (DGGE). Representatives of strains showing the same migration pattern were subjected to amplification of the V1-V3 regions of the 16S rRNA gene by using the primers P1V1 (5'- GCG GCG TGC CTA ATA CAT GC-3') and P4V3 (5'-ATC TAC GCA TTT CAC CGC TAC-3') as reported elsewhere (Cocolin *et al*. 2004). PCR products were purified by QIAquick® PCR purification kit (QIAgen, Milan, Italy) and sent for sequencing to MWG Biotech (Ebersberg, Germany). The retrieved sequences were aligned in Gene Bank by using the Blast program (http://blast.ncbi.nlm.nih.gov).

Targeting the bacteriocin encoding gene by PCR amplification

Genes encoding for the enterocins (A, P, B, L50A, L50B, AS-48, 31) were targeted by PCR using the primers and conditions as reported by De Vuyst *et al*. (2003). The reaction was performed in a final volume of 25 µl containing 1X PCR buffer, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTP, 0.2 mmol l^{-1} of each bacteriocin primer and 1 U of Taq polymerase (Applied Biosystem, Milan, Italy). The cycles used were 95°C for 5 min as initial step, 95°C for 1 min, 56°C (for the primers AS-48, *ent*A, *ent*P, *ent*B, L50A, L50B) or 52°C (for enterocin 31) for 1 min, and 72°C for 1 min for the next 30 cycles, 72°C for 5 min concluded the amplification. Fragments were visualized on 2% agarose gels added of ethidium bromide using a 100 bp ladder (Sigma, Italy) as molecular weight ladder. For each PCR, positive control strains from the Università di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari were used.

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Table 1 Sources of isolation, harbored enterocin genes and inhibitory spectrum of the enterococcal isolates used in this study.

Isolates	Origin	Identification	Enterocin genes	Spectrum of inhibition							
				L. monocytogenes	*L. innocua*	*L. ivanovii*	*Ent. faecalis*	*E. coli*	*S. aureus*	*S. enteritidis*	*P. larvae*
Ent B11	Feces of cows	*Ent. faecium*	A, P, L50A/B	++++[a]	+++	+++	++++	-	+++	-	-
Ent B10			A, P	-	+	++	-	-	-	+	-
Ent KA18			A, P, L50A/B	-	+	-	-	+	-	-	-
Ent K1			A, P	-	+++	-	++	+	-	-	-
Ent KA1			A, P	+	+++	++	-	+	-	-	+
Ent KA15			A, P	++	-	++	-	+	-	-	++
Ent S9			A	-	-	++	-	++	++	-	++
Ent SB8			A, P	++++	++++	++	-	-	-	-	++
Ent CH4			A, P, L50A/B	+++	-	+	-	-	-	-	++
Ent Bi28			P	+++	++++	++	++++	-	+++	-	++
Ent VF			A, P, L50A/B	-	+	+	-	-	-	-	-
Ent KA22			A, P	+++	+++	+++	+	+	-	+	-
Ent 102			A, P	+	+++	-	+++	-	-	-	+++
Ent KA24	Feces of sheep		A, P	-	++	+	-	+	-	-	-
Ent M7			A, P, L50A/B	-	-	+	-	-	-	-	+
Ent Bi5			A, P, L50A/B	++	+++	-	+++	-	++	-	+
Ent C2	Feces of		A, P, L50A/B	+++	+++	++	++	++	-	-	-

Ent P4	poultry		A, P, L50A/B	+	++	+	++	-	-	-	-
Ent 97			A, P	-	+++	+	+++	-	-	-	++
Ent P10			A, P, L50A/B	+	++	-	++	-	+	-	+
Ent 6			A, L50A/B	-	+++	-	-	-	-	-	+++
Ent 11			A, L50A/B	-	+++	+	-	-	-	-	-
Ent 12			A	+	+++	+	+++	-	-	-	+++
Ent 23			A, P, L50A/B	+	+++	+	+++	-	-	-	-
Ent 75			A	-	+	-	-	-	-	-	+++
Ent 76			A	+	+	+	-	-	-	-	+++
Ent 13			A	-	+++	+++	+++	-	-	-	+++
Ent 14			A	+	+++	+	+++	-	-	-	+++
Ent 59			A, L50A/B	+++	+++	+++	+++	-	-		+++
Ent OR42	Feces of bird		A, P, L50A/B	++	++	++	-	++	+	-	+
Ent 112	Swab from cat ear		A	+++	+++	+++	++++	-	-	-	++++
Ent 111	Swab from dog ear		A	++	+++	++	++++	-	-	-	+++
Ent L5	Raw milk		A, P, L50A/B	++++	++++	-	++++	-	++++	-	+++

Ent R1.2	Ricotta cheese		A, P, L50A/B	-	+	-	-	-	-	-	-
Ent K4	Feces of cows	*Ent. faecalis*	A	++	+	+	-	-	-	-	-
Ent S2			A, P, L50A/B	-	++	++	-	+	-	+	+
Ent KA20			A, P	+	++	+	+	-	+	-	++++
Ent KA16			A, P	++	++	-	++	-	-	-	+++
Ent KA8			A, P, L50A/B	++	++	++	-	++	-	-	++
Ent K3			A, P, L50A/B	-	+	+	-	-	-	-	-
Ent KAA	Feces of donkey		A, P, L50A/B	-	+	-	-	+	-	+	-
Ent M11	Feces of sheep		A, P, L50A/B	+	-	++	++	-	-	-	++
Ent KA26			A	+++	-	-	-	+	-	-	-
Ent C3	Feces of poultry		A, P, L50A/B	+++	+++	+++	+++	++	-	-	-
Ent P2			A, L50A/B	+	+++	++	-	+++	-	-	-
Ent P8			A, P, L50A/B	-	+	+	-	+	-	-	+++
Ent 20			ND[b]	-	+	-	-	-	-	-	+++
Ent 7			A	+	++	-	-	-	-	-	+++
Ent 8			A	+	+++	-	+++	-	-	-	+++
Ent 98			L50A/B	++	+++	-	+++	-	-	-	+++
EntOR30	Feces		A, P,	+++	+++	++	+++	-	+++	-	+++

	of bird		L50A/B								
Ent SP12	Water		A	+	+	+	-	-	++	++	-
Ent L4	Raw milk		A, P	+++	++++	++++	+++	-	-	-	-
Ent B3	Feces of cows	*Ent. hirae*	A, P	+	++	+++	+	-	-	++	-
Ent S7			A, P	-	++	+++	+	-	-	++	++
Ent SP13	Water		P	+	-	++	-	-	-	-	+++
Ent K2	Feces of cow	*Ent. mundtii*	A, P, L50A/B	+	+	+	-	-	-	-	-
Ent KA23	Feces of sheep		A	-	+	+	-	-	-	-	-
Ent SB3	Feces of cow	*Ent. durans*	A, P	++	++	++	++	-	-	-	++

[a]scores were assigned based on the diameter of the inhibition halo: ++++= clear zone >15mm, +++ = clear zone 10-15 mm, ++:5-9.9 mm, + = clear zone 1-4.99 mm, - = no zone.

[b]ND, not detected: no amplification signal was obtained by using the bacteriocin primers used in this study.